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Nonanticoagulant Heparins

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14. ABSTRACT A mouse model of breast cancer with human breast cancer cell lines MCF7 (wild type) or MCF7-doxorubicin resistant (MCF7-R) cells was used evaluate the efficacy of low molecular weight heparins (LMWH) either alone or in combination with doxorubicin to prevent tumor growth. Tumor volume measurements were performed at intervals throughout the course of treatment. LMWH compounds (Enoxaparin or non-anticoagulant heparin NACH) given together with chemotherapeutic agent doxorubicin decreased tumor growth rate and prolong survival in animals bearing MCF7 wild-type tumors. These agents appeared to be less effective in animals bearing doxorubicin-resistant tumors. Bleeding times determined on animals in all treatment groups showed that there were no statistically significant differences among the groups. However, animals in ENOX groups showed increased bruising at the sites of injection. These studies will be repeated, and studies with alpha v beta 3-targeted nanoparticle formulations will be performed to compare the efficacies of non-targeted and targeted therapies.					
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INTRODUCTION:

A broad spectrum of clinically significant hemostatic abnormalities may afflict as many as 15-25% of cancer patients. Furthermore, hemostatic complications are the second most common cause of mortality in cancer patients particularly in those with pancreatic, gastrointestinal or lung cancer, and 10% of newly diagnosed myeloma patients treated with any type of chemotherapy develop deep venous thrombosis (1-3). There is substantial literature support for the use of low molecular weight heparin (LMWH) for treating coagulation disorders in cancer patients. However, recent prospective clinical trials have demonstrated that they provide significant advantages in terms of progression-free and overall survival in certain cancers and in certain subgroups of patients (4-8). Data from *in vitro* and experimental animal models also provide encouraging scientific rationales for application of these agents to control tumor growth and metastasis (9-12). Survival advantages have not been seen in breast cancer trials, perhaps because increased bleeding times in these patients constitute a dose-limiting side effect. We have developed novel non-anticoagulant heparin (NACH) compounds that have minimal effects on hemostasis (13). In the studies proposed, we will test the ability of NACH to improve the efficacy of chemotherapy treatment without affecting hemostasis. In some studies, we will use PEG-PLGA nano-particles for targeted drug delivery of NACH and Doxorubicin (DOX), directing therapeutic treatments to the tumor neovasculature by attaching alpha v beta 3 antibody to the surface of nano-particles. New nano-particle technology provides unprecedented opportunities for addressing areas in breast cancer research due to the utilization of biodegradable/biocompatible polymeric materials for carrying therapeutic agents to tumor sites. Nano-therapy studies have just begun in man and experimental studies such as the one proposed here will provide support for application of such regimens for the treatment of breast cancer in the future and advance research in this field. These studies will be performed in a mouse orthotopic model of breast cancer using Doxorubicin-sensitive or –resistant MCF7 human breast cancer cells.

BODY: RESEARCH ACCOMPLISHMENTS DURING YEAR 1

Statement of work

The research studies to be performed during the first year of this grant are summarized in **Specific Aim 1**: Female athymic mice will have either drug-resistant or drug-sensitive MCF7 human breast cancer cells implanted orthotopically into the fourth mammary gland. Treatment modalities will be evaluated for their effects on tumor growth, metastasis and tumor-associated angiogenesis, and will include nano-particle targeted vs. un-targeted therapies as outlined below. Bleeding times will be performed in a cohort of animals to confirm that NACH treatments have minimal effects on hemostasis in these tumor-bearing animals. Evaluations will include determinations of the size of tumors, quantification of metastases and tumor-associated angiogenesis. For evaluation of metastases, lungs will be removed from the thoracic cage *en bloc* and lung seeding will be assessed by counting macroscopically the number of pulmonary tumor nodules on the entire surface of the lungs, and microscopically by histology of lung sections. Bleeding times will be performed using standard methodology. Tumor-associated angiogenesis will be evaluated using endothelial cell-specific CD31/PECAM staining.

Treatment Groups

1. Controls: no treatment
2. Doxorubicin (DOX) alone
3. DOX + Enoxaparin (ENOX)
4. DOX + LMWH compound NACH
5. Control nanoparticle: without surface targeting and containing no therapeutic treatment
6. Targeted nanoparticle + DOX
7. Targeted nanoparticle + DOX + Enoxaparin
8. Targeted nanoparticle + DOX+ NACH

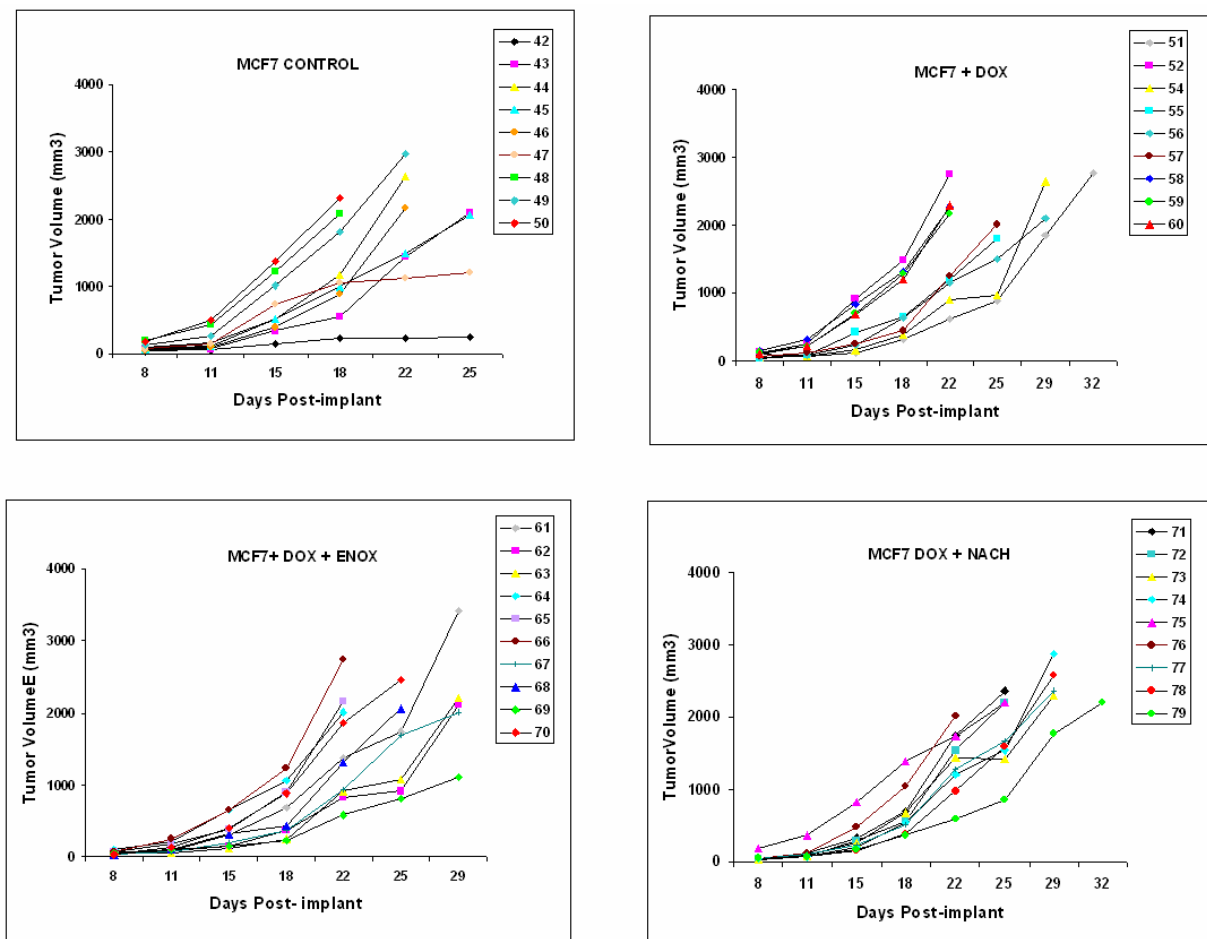
Experimental Design

- Tumor cell lines MCF7 – wild type or MCF7-R (DOX-resistant) were injected into 4th mammary fat pad of nude mice.
- Animals were randomized into treatment groups 7 days after tumor implant when tumors were palpable or 50 mm³ in size. Treatments were begun.
- Treatments: DOX 2.5 mg/kg SC injection 3x/week (Monday, Wednesday Friday); Enoxaparin or NACH 10 mg/kg 5x/week (Monday – Friday); For combination therapy: 2.5 mg/kg DOX + either 10 mg/kg Tinzaparin OR NACH.
- Bleeding times were determined 3 hrs post initial dose of either Enoxaparin or NACH. Each animal was subjected to bleeding time testing only once in the course of the experiment.
- Tumor measurements were obtained at 3-4 day intervals, starting Day 8 after tumor implantation.
- Animals were sacrificed tumor weights obtained.
- Tumors and lungs were fixed for histology and immunohistochemistry to evaluate tumor-associated angiogenesis.
- Body cavities were examined for the presence of metastases and observations recorded.

Results

To date, only the first half of the study has been performed – non-nanoparticle targeted treatments. Nanoparticles have been prepared and will be utilized for the second half of the study.

Figure 1: Tumor volume plots MCF7 tumors. Plots are shown for individual animals implanted with MCF7 (wild type) tumor cells and treated as designated on each plot and described above. Tumor measurements were begun at 8 days post-post implant and continued every 3-4 days until animals were euthanized.



Data show that DOX-treated animals showed improved survival but here was variability in animal responses in both this treatment group and in DOX + ENOX groups. However, animals in the DOX + NACH group showed a more unified responses, with clusters of animals showing similar tumor growth rates. To quantify these data, we evaluated the time in Days to form tumors 2000 mm³ in size. Comparison of treatments for anti-tumor efficacy is shown below in Table 1 below. Data are also summarized with respect to number of animals surviving at 2 separate time points, Day 25 and Day 29.

Table 1 Anti-tumor efficacy of LMWH treatment in combination with DOX in nude mice with MCF7 tumors

Treatment group ^a	Days to 2000 mm³ Mean \pm SEM (range)	P value ^b (Control)	P value ^c (DOX)	Surviving animals Day 25	Surviving animals Day 29
Control	20.57\pm 1.17 (17-25)	-	0.04	3/8	0/8
DOX	24.75\pm 1.46 (21-31)	0.04	-	5/9	3/9
DOX + ENOX	25.11\pm1.18 (21-29)	0.016	0.850	7/10	5/10
DOX + NACH	26.33 \pm1.03 (22-32)	0.003	0.39	8/10	5/10

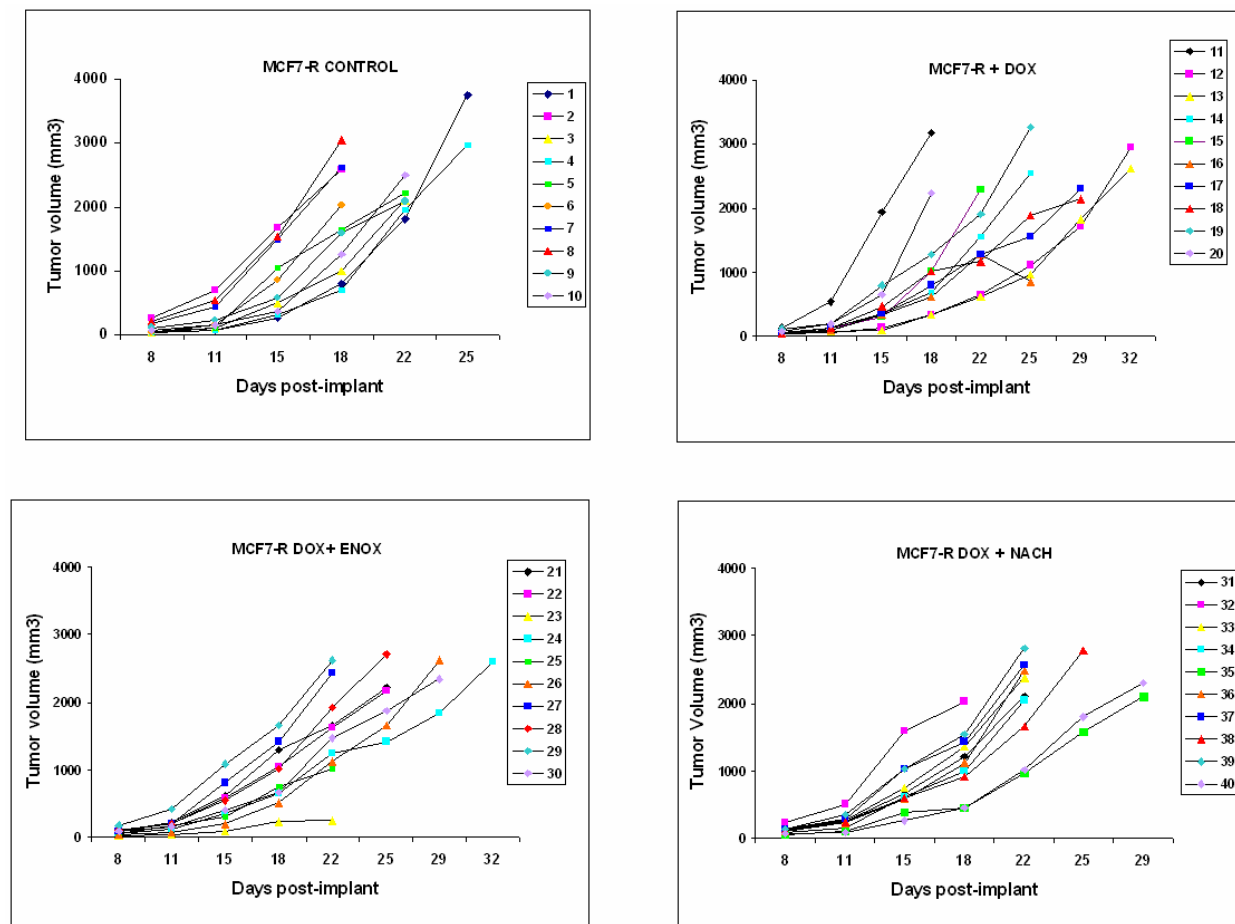
^a Treatment of nude mice beginning day 8 after implant of MCF7 tumor cells

^b based on comparison of each group vs. control using two-tailed t-test

^c based on comparison of each group vs. DOX using two-tailed t-test

Data in Table 1 demonstrate that DOX treatment resulted in a statistically significant increase in the time for tumors to reach 2000 mm³ and resulted in increased survival of the animals in comparison to untreated control groups. Both DOX + ENOX and DOX + NACH groups significantly attenuated tumor growth to 2000 mm³, even though the differences between these groups and DOX did not reach statistical significance. In addition, animal survival in these groups was improved relative to animals receiving DOX alone. The increased survival ratios and lengthening of the time required for tumor growth indicate that these treatments may have the potential for increasing the efficacy chemotherapeutic agents and should be pursued. These studies will be repeated to confirm the findings and increase the likelihood of attaining statistical significance in comparison to DOX alone treatments.

Figure 2: Tumor volume plots MCF-R tumors. Plots are shown for individual animals implanted with MCF7-R doxorubicin-resistant tumor cell line and treated as designated on each plot and described above. Tumor measurements were begun at 8 days post-post implant and continued every 3-4 days until animals were euthanized.



Data show that all three treatments provided some survival advantage in comparison to untreated animals. DOX treatment alone prolonged survival and in some animals may have decreased the rate of tumor growth, although considerable variation in response was observed in this group. In DOX+ ENOX group, the response to treatment appeared to be more consistent than in the DOX group. In the DOX + NACH group only 3/10 animals survived at Day 25 and 2/10 at Day 29 suggesting that this treatment did not improve the efficacy of DOX alone. In this group data were clustered in the time points encompassing 8-22 Days. However, two animals in this treatment group showed decreased tumor growth rate and increased survival. The survival data for these animals (Table 2 below) in the DOX + ENOX and DOX + NACH groups is different from that observed in Table 1 for animals bearing the MCF7 tumors.

To quantify these data, we evaluated the time in Days to form tumors 2000 mm³ in size. Comparison of treatments for anti-tumor efficacy is shown below in Table 2 below. Data are also summarized with respect to number of animals surviving at 2 separate time points, Day 25 and Day 29.

Table2: Anti-tumor efficacy of LMWH treatment in combination with DOX in nude mice with MCF7-R tumors

Treatment group ^a	Days to 2000 mm³ Mean \pm SEM (range)	P value ^b (Control)	P value ^c (DOX)	Surviving animals Day 25	Surviving animals Day 29
Control	20.1 \pm 0.81 (16-23)	-	0.050	2/10	0/10
DOX	24.56 \pm 1.85 (15-32)	0.050	-	7/10	4/10
DOX + ENOX	24.37 \pm 1.25 (21-29)	0.014	0.936	6/10	3/10
DOX + NACH	22.5 \pm 1.18 (18-29)	0.11	0.366	3/10	2/10

^a Treatment of nude mice beginning day 8 after implant of MCF7-R tumor cells

^b based on comparison of each group vs. control using two-tailed t-test

^c based on comparison of each group vs. DOX using two-tailed t-test

Data demonstrate that both DOX and DOX + ENOX treatments significantly increase the time interval to the development of tumors sized 2000 mm³, while DOX + NACH group shows less effective protection with a P value approaching but not reaching statistical significance. This observation is corroborated by comparison of the number of surviving animals in DOX and DOX + ENOX categories. These studies will be repeated for confirmation, and because of the variability of responses, repeat studies may allow us to determine whether there are statistically significant differences among treatment groups.

Table 3 Bleeding time studies performed on all treatment groups. Bleeding times were

BLEEDING TIME MCF7 ANIMALS			
CONTROL	Mean	GROUP A	GROUP B
	SD	0:40	2:12
DOX	Mean	1:46	1:24
	SD	1:17	0:12
DOX + ENOX	Mean	3:44	1:40
	SD	3:28	0:20
DOX + NACH	Mean	1:54	1:30
	SD	1:58	0:27
BLEEDING TIME MCF7- R ANIMALS			
CONTROL	Mean	GROUP A	GROUP B
	SD	1:32	1:23
DOX	Mean	1:32	0:33
	SD	1:59	1:21
DOX + ENOX	Mean	1:30	0:10
	SD	3:05	1:11
DOX + NACH	Mean	1:44	0:18
	SD	2:28	3:13
		1:49	3:02

determined by standard methodology (14) to evaluate whether treatment with LMWH would increase this indicator of disrupted hemostasis. Because each animal was subjected to bleeding time testing only once in the course of the experiment, each treatment group consisted of sub-groups A and B (5 animals per sub-group), total n per treatment group = 10. Table 3 summarizes the bleeding time data expressed as minutes and seconds \pm SD. Although there was a trend toward increased bleeding time in DOX + ENOX groups, for both MCF7 and MCF7-R groups, there were no statistically significant differences between the ENOX groups and the other groups. However, approximately half of the animals in ENOX groups had bruising at the sites of injection.

KEY RESEARCH ACCOMPLISHMENTS: (bulleted list)

- Experiments for the first part of Specific Aim 1 (non-nanoparticle agents) have been performed. Data are presented in this report
- Bleeding tests for these studies have been performed. Data are presented in this report.
- Tumor and lung tissue has been obtained from all animals and is being processed for examination. Data will be presented when complete.
- Nanoparticle formulations for the second part of Specific Aim 1 have been prepared and characterized. They are ready for use in targeted-nanoparticle agent studies

REPORTABLE OUTCOMES:

- An abstract has been accepted for presentation as a poster at the Era of Hope Meetings in June 2008. The data in this report and additional studies performed as an outgrowth of the concepts supported in this grant will be presented.
- Data from this study, when complete, will be submitted for publication.
- Collaborative studies are underway with a group at Roswell Park to pursue the therapeutic potential of LMWH in cancer, specifically their effects on uptake of chemotherapeutic agents. These studies, supported by a Phase I SBIR grant, have potential for submission for a Phase II grant.

CONCLUSIONS: LMWH compounds given together with chemotherapeutic agent doxorubicin decreased tumor growth rate and prolong survival in animals bearing MCF7 wild-type tumors. These agents appear to be less effective in animals bearing doxorubicin-resistant tumors. Bleeding times determined on animals in all treatment groups did not show statistically significant differences. However, animals in ENOX groups showed increased bruising at the sites of injection. These studies will be repeated, and studies with alpha v beta 3-targeted nanoparticle formulations will be performed to compare the efficacies of non-targeted and nanoparticle-targeted therapies.

APPENDICES:

The Abstract to be presented at the Era of Hope Meeting in June 2008 is included in the Appendix.

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